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(54) Title: INHIBITION OF ATTACHMENT OF <i>H. INFLUENZAE</i> TO HUMAN CELLS			
(57) Abstract			
<p>The attachment of <i>H. influenzae</i> to human cells, such as oropharyngeal cells, may be inhibited by native human β-casein, a recombinant form of human β-casein, and hydrolysates of both. The human β-casein or hydrolysate may be contained in a liquid enteral nutritional product such as an infant formula. The enteral nutritional product may be used, for example, in the prevention and treatment of otitis media in infants. The human β-casein or hydrolysate may also be administered as a throat spray or nasally using drops or a spray.</p>			

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INHIBITION OF ATTACHMENT OF H. INFLUENZAE TO HUMAN CELLS

The present invention relates generally to inhibiting the attachment of *Haemophilus influenzae* to human cells, and more specifically to the use of native or recombinant human β -casein and hydrolysates thereof for inhibiting the attachment of *Haemophilus influenzae* (*H. influenzae*) to human cells.

Haemophilus are small, gram-negative, non-motile, non-spore forming bacilli with complex growth requirements. Diseases caused by *H. influenzae* usually begin as a nasopharyngitis, possibly precipitated by a viral infection of the upper respiratory tract. Morse, et al. "*Haemophilus*", MICROBIOLOGY, FOURTH EDITION, published by J.B. Lipincott Company, pages 615-618 (1990).

H. influenzae are spread from person to person by airborne respiratory droplets or direct contact with secretions. To colonize, *H. influenzae* must contend with ciliary clearance mechanisms of the nasopharyngeal mucosal surface and the mucous barrier. Once past the mucous barrier and the ciliary escalator, *H. influenzae* attach to mucosal epithelial cells. Invasion of mucosal surfaces appears to be an important characteristic of pathogenic bacteria. Stephens, et al., "Pathogenic Events During Infection of the Human Nasopharynx with *Neisseria meningitis* and *Haemophilus influenzae*", REVIEWS OF INFECTIOUS DISEASES, 13:22-23 (1991). It has further been reported that *H. influenzae* harbored in the nasopharynx are a key factor in the development of middle ear infections (otitis media), and that non-typable *H. influenzae* adhere to nasopharyngeal and nasal mucosal cells. Harada et al., "Adherence of *Haemophilus influenzae* to nasal, nasopharyngeal and buccal epithelial cells from patients with otitis media" EUROPEAN ARCHIVES OF OTO-RHINO-LARYNGOLOGY, 247:122-124 (1990). Stenfors et al., "Abundant Attachment of Bacteria to Nasopharyngeal Epithelium in Otitis-Prone Children", THE JOURNAL OF INFECTIOUS DISEASES, 165:1148-1150 (1992). In accordance with the present invention β -casein isolated from human milk or a recombinant form thereof, or a hydrolysate of either is employed to inhibit the adhesion of *H. influenzae* to human cells.

Exclusive breast-feeding of human infants for the first four months of life has been associated with a decrease in the incidence of both acute

and recurrent otitis media. However, the question remained as to whether it is breast milk per se or the mechanics of breast feeding that exert the protective effect. Sheard, "Breast-Feeding Protects Against Otitis Media", NUTRITION REVIEWS, Vol. 51, No. 9, pages 275-277 (1993). In one study of 1,013 infants followed for their entire first year, 47% had at least one episode of otitis media and 17% had recurrent otitis media. Infants exclusively breast fed for four or more months had half the mean number of acute otitis media episodes as those not breast fed at all and 40% less than those infants whose diets were supplemented with other foods prior to four months. The recurrent otitis media rate in infants exclusively breast-fed for six months or more was 10% and was 20.5% in those infants breast-fed for less than four months. Those investigators presented speculations that IgA, or micronutrients or Prostaglandin E, in breast milk may be protective, but concluded that the mechanism for a protective effect of breast-feeding against otitis media was not clear. Duncan et al., "Exclusive Breast-Feeding for at Least 4 Months Protects Against Otitis Media", PEDIATRICS, Vol. 91, No. 5, pages 867-872 (1993).

WO 91/06308 filed by Andersson et al. for "ANTIBACTERIAL COMPOSITION", and a published article by the same authors (Aniansson et al., "Anti-adhesive activity of human casein against *Streptococcus pneumonia* and *Haemophilus influenzae*", MICROBIAL PATHOGENESIS, 8:315-323 (1990) disclose the use of a milk fraction having a molecular weight of at least 5,000 daltons for "therapeutic prophylactic, and/or diagnostic use in infections caused by *S. pneumoniae* and/or *H. influenzae*", but it is suggested in these publications that the beneficial effect is provided by kappa-casein. However, the present invention relates to the use of native or recombinant human β -casein and hydrolysates of both to inhibit *H. influenzae* infections.

WO93/04172 relates to a DNA sequence encoding human β -casein, but does not disclose the capacity of either native or recombinant human β -casein to inhibit the attachment of *H. influenzae* to human cells.

WO91/08675 discloses an infant formula which contains recombinant forms of both human alpha-lactalbumin and human β -casein. However, this publication discloses only that these human milk proteins will "give a simulated human mother's milk formula that does not exhibit the allergenic properties associated with formulas based on cow or other foreign protein." (page 3, lines 20-22). The use of human β -casein to inhibit the attachment

of *H. influenzae* to human cells is not taught or suggested in said publication.

The two assays (a radiolabeled assay and an ELISA assay) which were used for determining the bioactivity of β -casein are described below. These assays have not been published heretofore, although the ELISA assay was based upon established methodology.

MATERIALS USED IN BOTH ASSAYS

Haemophilus influenzae

Haemophilus influenzae (*H. influenzae*) cultures (fimbriated, nontypable) which have been implicated in otitis media were obtained from Dr. Lauren Bakeletz of The Ohio State University, Columbus, Ohio, U.S.A. The use of these organisms in assays has been described in Bakeletz, et al., "Frequency of Fimbriation of Nontypable *Haemophilus influenzae* and its Ability to Adhere to Chinchilla and Human Respiratory Epithelium". Infection and Immunity, 56: 331-335 (1988). The *H. influenzae* were streaked onto Chocolate agar plates (BBL-Becton Dickinson & Co., Cockeysville, Maryland, U.S.A.) from frozen aliquots of a low passage number and incubated at 37°C in a 5% CO₂ incubator for about 18 hours to obtain logarithmically growing cultures. The *H. influenzae* was used in both the Enzyme Linked Immuno Sorbent Assay (ELISA) assay and the radiolabeling assay as described below.

Native Human β -Casein

β -casein isolated from human milk was purchased from Symbicom AB, P.O. Box 1451, S-901 24 Umeå, Sweden.

Recombinant Human β -Casein

Applicants obtained β -casein cDNA and the expression system from Symbicom AB, P.O. Box 1451, S-901 24 Umeå, Sweden. The human β -casein cDNA used had been previously cloned and sequenced by Lonnerdal et al., Cloning and sequencing of a cDNA encoding human milk β -casein. (SEQ ID NO: 1:) *Federation of European Biochemical Societies Letters* 269, 153-156 (1990). The recombinant human β -casein was obtained from *E. coli* and purified according to the method of Hansson et al., Expression of Human Milk β -Casein in *Escherichia coli*: Comparison of Recombinant Protein with Native Isoforms.

Protein Expression and Purification 4, 373-381 (1993). To express human β -casein in *E.coli*, β -casein cDNA was cloned under control of a T7 promoter in two different expression vectors. One vector, pS26, was designed for intracellular expression. The other vector, pS28, has a signal sequence for extracellular expression. The procedure followed was substantially that described by Hansson et al.

Human β -casein cDNA was isolated by Hansson et al. as a 1.1-kb *EcoRI* fragment from a human lambda gt mammary gland library, and was subcloned into pUC19, which was designated pS21. The cDNA was modified by introduction of synthetic oligonucleotides in the 5' and 3' termini. To introduce a suitable cloning site in the 5' end, *NdeI*, a translational start, was inserted in front of the sequence encoding mature human β -casein. To adapt the initial part of the translated sequence to *E.coli* codon usage, six synthetic oligonucleotides were constructed and ligated. Also, *PstI* and *EcoRI* sites were inserted in front of the *NdeI* site. The sequence of the synthetic fragment was 5'-CTGCAGAATTCATATGCGTGAACCATCGAATCCCTGAGCTCGAGCGAAGAATCGATCACCGAATACAAAAAGTTGAAAAAGTTAAACACGAGGACCAGGATCC-3'. (SEQ ID NO: 2:) The protein encoding sequence is underlined. The synthetic fragment was cloned into *PstI/BamHI*-digested pUC19 resulting in plasmid pS24. To insert the rest of the β -casein encoding sequence, a 303-bp *AccI/BglII* fragment was isolated and cloned into a pUC18 derivative and designated plasmid pS22. Four synthetic oligonucleotides containing the sequence encoding the carboxy-terminal end and translation stop followed by *BamHI* and *EcoRI* sites were constructed resulting in the sequence 5'-AGATCTACCCCTGTGACTCAGCCACTTGCCCCAGTTCATAACCCCATTAGTGTCTAATAAGGATCCGAATTC-3'.

(SEQ ID NO: 3:) where the protein encoding sequence is underlined. The synthetic fragment was cloned into *BglII/EcoRI* digested pS22, resulting in plasmid pS23. To obtain the recombinant modified β -casein encoding fragment, three fragments were ligated: an 89-bp *PstI/AvaII* fragment from pS24; a 197-bp *AvaII/AccI* fragment from pS21; and *PstI/AccI* digested pS23. The resulting plasmid pS25 was digested with *NdeI/BamHI* and a 641-bp fragment was isolated and cloned into the vector pET-3a. The resulting expression vector was designated pS26.

In order to construct a vector mediating extracellular expression, the *E. coli* signal sequence of the enterotoxin STII gene was introduced in front

of the β -casein encoding sequence. A modified STII sequence with *Nco*I- and *Nde*I-compatible ends and an internal *Cla*I site was obtained by using a synthetic oligonucleotide, 5'-CATGAAAAGAATATCGCATTTCTTCTTGCATCGATGTTTCGTTT TTTCTATTGCTACAAATGCATATG-3' (SEQ ID NO: 4:). To insert the signal sequence in front of the β -casein encoding sequence, pS25 was digested with *Ava*I/*Eco*RI and a 619-bp fragment was isolated. This fragment was ligated with a synthetic oligonucleotide fragment, 5'-CATATGCACGTGAAACCATCGAATCCCTGAGCTCGAG-3' (SEQ ID NO: 5:), and *Nde*I/*Eco*RI-digested pUC19. The resulting plasmid was designated pS27. The final expression vector, pS28, was constructed by ligating three fragments: a 700-bp *Nde*I/*Hind*III β -casein fragment isolated from pS27, the STII signal sequence, and a *Nco*I/*Hind*III-digested pACAT7 vector.

The expression vectors pS26 and pS28 were used to transform *E. coli* strains BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)pLysE. The bacteria were grown in Luria Broth medium containing 50 μ g/ml carbenicillin, and when BL21(DE3)pLysS and BL21(DE3)pLysE were used the medium was supplemented with 25 μ g/ml chloramphenicol.

For induction of expression the cultures were grown to a density yielding an optical density (OD) of 0.6 at a wavelength of 600 nanometers (OD_{600}), then 0.4 mM IPTG was added to induce the T7 system. The cells were harvested about 90 minutes after induction.

Recombinant β -casein was isolated using standard procedures. The inducible T7-based expression system resulted in high-level expression of recombinant β -casein. Bacteria were harvested and the cells pelleted by centrifugation. The supernatant contained the periplasmic proteins and the pellet the cytoplasmic fraction. The recombinant proteins obtained were compared with native β -casein, which had been purified by standard methods including either ion-exchange chromatography followed by reversed-phase HPLC or gel filtration. Recombinant and native β -casein were compared by standard biochemical techniques comprising SDS-PAGE, Western blotting, amino acid analysis, peptide mapping, phosphate analysis, and mass spectrometry. Recombinant β -casein expressed in *E. coli* was found to comigrate with full-length, nonphosphorylated native human β -casein, which is one of seven native isoforms.

Recombinant human β -casein has also been expressed in *S. cerevisiae* using the pYES 2.0 vector (Invitrogen Corp., San Diego, CA), but the

expression level was approximately 10% of that obtained in *E.coli*. However, Hansson et al. found that *S. cerevisiae* appeared to express phosphorylated human milk β -casein.

β -Casein Hydrolysates

The human β -casein (both native and recombinant) was digested using the specific endoproteinase GLU-C (Sigma, sequencing grade) which catalyzes the hydrolysis of peptide bonds at the C-terminal of glutamic acid residue. After monitoring the digest using high pressure liquid chromatography, an enzyme to protein ratio of 1:100 (weight/weight) was chosen for a 30 hour digestion at 37°C in 0.1 M NH_4HCO_3 , pH 7.8. These digests were dried and resuspended in appropriate buffers prior to use in the assays discussed above.

RADIOLABELED ASSAY

Detroit 562 cells

Detroit 562 pharyngeal carcinoma cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The use of this type of cell in assays has been described in Takahashi, et al. "Phosphorylation Of A Surface Receptor Bound Urokinase-Type Plasminogen Activator In a Human Metastatic Carcinomatous Cell Line", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, 182:1466-72. The Detroit 562 cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, New York, U.S.A.) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, Utah, U.S.A.) Cells were routinely subcultured in 75 cm² flasks (Costar, Cambridge, Massachusetts, U.S.A.) using Trypsin-EDTA (0.25% trypsin, 1mM EDTA (Ethylenediaminetetraacetic acid), (GIBCO) to detach cells. Cell subculture passages 48-75 were utilized for adhesion studies. Cells were seeded into 96 well plates (Costar) at a density of 20,000 cells per well and maintained at 37°C in a 5% CO₂ incubator for 8-10 days providing a confluent monolayer for adhesion studies. Plates were washed three times with 200 µl Hanks Balanced salt solution (HBS) (GIBCO), supplemented with 30 mM of N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid (HBS) (GIBCO) to remove serum proteins, before addition of bacteria in adhesion assays.

Radiolabeling of bacterium

Harvested bacteria in phosphate buffered saline supplemented with 0.05% Bovine serum albumin (PBS-BSA) were centrifuged and resuspended in a volume of PBS-BSA yielding an optical density (OD) of 2.4 at a wave length of 600 nanometers (OD₆₀₀). ¹¹¹-Indium-oxine (¹¹¹-In) (Amersham, Arlington Heights, Illinois, U.S.A.) a high energy, short lived tracer, was utilized to radiolabel the bacteria. (The ¹¹¹-In penetrates the cell membrane, where once inside the cell it dissociates resulting in the binding of ¹¹¹-Indium ions with cytoplasmic components.) The use of ¹¹¹-In labeling in other assays has been described in Ardehali, et al. "¹¹¹-Indium Labeling of Microorganisms to Facilitate the Investigation of Bacterial Adhesion", JOURNAL OF BIOMEDICAL MATERIALS RESEARCH, 27: 269-275 (1993). 50 µCi of an ¹¹¹-In solution was added to 2.5 ml of the bacterial suspension at 37°C and

incubated for 20 minutes. The radiolabeled bacteria were then washed two times and resuspended in 5 ml HBS supplemented with 30 mM HEPES buffer. 25 μ l of the 111 -In labeled bacterial suspension were preincubated with 25 μ l of β -casein in a polypropylene 96 well plate for 15 minutes at 37°C to allow binding of β -casein to bacteria.

Radiolabeled bacterial adhesion measurement

25 μ l of the preincubation mixture containing both radiolabeled bacteria and β -casein was pipetted into each well of the assay plate containing Detroit 562 cells. The assay plate was incubated for 20 minutes at 37°C to allow adhesion of the bacterium to the cell monolayer. The plates were then washed three times with HBS to remove nonadhering bacteria from the Detroit 562 cells. The assay was terminated by the addition of 100 μ l of 1 N sodium hydroxide (NaOH) to disrupt the cell monolayer. The entire contents of each well was placed in a Cobra polypropylene tube (12 mm in diameter and 75 mm in height) (Packard, Meriden, Connecticut, U.S.A.) and counted on a Cobra gamma counter (also from Packard). Results were calculated by averaging the results of four replicates after the subtraction of background radiation. Results are presented as percent inhibition of bacterial attachment in no agent control wells.

RESULTS FROM RADIOLABELED ASSAY

The human and bovine β -casein solutions were prepared first in 20 mM ethanolamine, 6 M urea, pH 9.5 and then washed twice in PBS by ultrafiltration using Centricon membrane filters (Amicon, MA) with a cut-off of 3,000 daltons. After resuspending in appropriate buffer for the radiolabeled assay described above, these samples were tested in the assay. Experiments with different designated numbers were performed in different days. As shown in Table 1, human β -casein exhibited an inhibition of 50% or more at concentrations of 0.75 mg/ml or greater. It should be noted that when referring to Table 1, a higher percent inhibition indicates a higher level of bioactivity of the "AGENT", and a lower percent inhibition indicates a lower level of activity of the "AGENT". Bovine β -casein was not significantly active even at 1.5 mg/ml. These results indicated that β -casein from human milk has different bioactivity compared to the bovine milk β -casein.

Hydrolysate of human β -casein obtained with GLU-C enzyme (prepared as described above) was also active (>50% inhibition) at concentrations of 0.75 mg/ml or higher. When the GLU-C hydrolysate of purified recombinant β -casein was tested at 3.0 mg/ml, it exhibited activity similar to that of the human milk β -casein hydrolysate. Hence, this recombinant protein could be produced in large-scale from bacteria to provide an abundant supply of a protein which retains the anti-adhesion activity of native human milk β -casein against *H. influenzae*.

TABLE 1
PERCENT INHIBITION OF *H. influenzae* ATTACHMENT
TO DETROIT 562 CELLS

<u>AGENT</u>	<u>CONCENTRATION</u> <u>MG/ML</u>	<u>EXP 1</u>	<u>EXP 2</u>	<u>EXP 3</u>	<u>AVERAGE</u>
β -Casein Isolated from Human Milk	3	67	ND*	ND*	67
	1.5	71	34	45	50
	0.75	60	ND*	ND*	60
	0.5	ND*	27	28	28
Hydrolysate of β -Casein Isolated from Human Milk	3	60			
	1.5	51			
	0.75	52			
Recombinant Human β -Casein Hydrolysate	3.0	71			
Bovine β -Casein	1.5	31			
	0.5	24			

* ND = NOT DONE

ELISA ASSAY

H. influenzae Preparation

Harvested *H. influenzae* from chocolate agar plates were suspended in 7 ml PBS-BSA and biotinylated by incubation with 350 μ l of biotin-caproate N-hydroxysuccinimide ester (0.01 g biotin/1ml dimethyl sulfoxide; Sigma, St. Louis, Missouri, U.S.A.) for 15 minutes at room temperature. The biotinylated bacteria were washed three times at 2,700 rpm for 30 minutes each to remove excess biotin. The labeled bacteria were then resuspended at an OD₆₀₀ of 2.4 using PBS-BSA.

Oropharyngeal cells

Oropharyngeal (OP) cells were collected from donors and pooled in phosphate buffered saline (PBS). Cell suspensions washed one time in PBS were resuspended and counted using a hemocytometer. Cells were adjusted to a density of 2.5×10^5 cells per ml in PBS. To promote OP cell attachment, 96-well plates (Linbro-ICN, Costa Mesa, California, U.S.A.) were coated with L-lysine followed by exposure to 1.25% glutaraldehyde (creates cross-linking). Plates were thoroughly washed to remove any residual glutaraldehyde. Each well of the 96-well plate was inoculated with 50 μ l of the OP cell preparation yielding a final concentration of 1.25×10^4 cells per well. Designated wells were incubated with PBS only (no OP cells) to serve as background control wells to measure non specific bacterial binding to plastic. Inoculated plates were centrifuged at 2,700-3,000 rpm for ten minutes to sediment the suspended cell preparation, aspirated and incubated overnight at 37°C in a moist chamber. The next morning plates with OP cells were treated with PBS containing 5% BSA for four hours to prevent non-specific bacterial attachment. The plates were washed three times with 200 μ l PBS-BSA before use in adhesion assays.

Adhesion Measurement

The biotinylated *H. influenzae* were diluted 1:1 with β -casein or control buffer (PBS-BSA) and incubated at 37°C in a shaking water bath for 30 minutes. 50 μ l of the preincubation mixture was placed into the appropriate well of the OP cell assay plate and incubated for 60 minutes at

37°C. The assay was halted by washing the assay plates three times with PBS-BSA and the plate heat fixed at 65°C for 10 minutes. After the plate cooled to room temperature, 100 μ l of Extravidin-peroxidase conjugate (Sigma), diluted 100-fold in PBS-BSA, was added to each well and the plate incubated for 40 minutes at 37°C. This conjugate binds to the biotin-labeled bacteria. Excess unbound conjugate was removed by washing the assay plate three times with PBS-BSA and 100 μ l of peroxidase substrate 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) was added to each well. Plates were incubated for 10 minutes and subsequently monitored for color development on a Thermomax 96 well plate reader (Molecular Devices, Menlo Park, California, U.S.A.) until the positive control wells containing OP cells and biotinylated bacteria (no β -casein) reached an OD₆₀₀ of 2.5 to 3.0. Binding results were calculated by averaging the results of three replicates.

RESULTS FROM ELISA ASSAY

Since the Detroit 562 cells were derived from a pharyngeal tumor, the proteins described in Table 1 were also tested in an anti-adhesion assay with normal human oropharyngeal cells from volunteers. Results from this ELISA assay are shown in Table 2. Once again, native human milk β -casein and recombinant human β -casein were found to be active at 0.5 mg/ml while bovine β -casein was inactive in the experiments described in SET I. After the analysis of the readings was adjusted for maximum sensitivity of the assay, human β -casein and its GLU-C hydrolysate were still active at the concentrations of 0.5 mg/ml and above (SET II). Hence these results indicated that the recombinant human β -casein, human milk β -casein and its hydrolysate inhibit attachment of *H. influenzae* to normal human oropharyngeal cells as well as human tumor cells.

TABLE 2
PERCENT INHIBITION OF *H. influenzae* ATTACHMENT
TO HUMAN OROPHARYNGEAL CELLS

SET I	AGENT	CONCENTRATION MG/ML	EXP 1	EXP 2	EXP 3	EXP 4	EXP 5	AVERAGE
			<u>EXP 1</u>	<u>EXP 2</u>	<u>EXP 3</u>	<u>EXP 4</u>	<u>EXP 5</u>	<u>AVERAGE</u>
	β -Casein Isolated from Human Milk	0.5	10	45	56	45	31	37
	Recombinant Human β -Casein	0.5	49	58	53	40		50
	Bovine β -Casein	0.5	0	0	0	0	0	0
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SET II	AGENT	CONCENTRATION MG/ML	EXP 1	EXP 2	EXP 3	AVERAGE		
			<u>EXP 1</u>	<u>EXP 2</u>	<u>EXP 3</u>	<u>AVERAGE</u>		
	β -Casein Isolated from Human Milk	1	53	88	68	70		
		0.5	12	ND*	82	47		
		0.25	20	ND*	ND*	20		
	Hydrolysate of β -Casein Isolated from Human Milk	1	70	48				
		0.5		73				

* ND = Not Done

It has been concluded from the foregoing experiments that β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk, and hydrolysates of both, inhibits the attachment of *H. influenzae* to human cells. Furthermore, inasmuch as *H. influenzae* have been identified in the literature as being associated with otitis media, it has been concluded that the above identified forms of human β -casein may be employed in the prevention and treatment of otitis media in humans, especially in human infants. In view of the therapeutic effect of enterally ingested human milk, containing human β -casein upon otitis media, it is concluded that the above identified forms of human β -casein have a therapeutic benefit when enterally (orally) ingested.

The therapeutic effects described in the preceding paragraph may be provided by an enteral liquid nutritional product, such as infant formula, comprising one or more proteins not contained in human milk in combination with a therapeutically effective amount of at least one of the forms of human β -casein described in the preceding paragraph. It is further concluded that the attachment of *H. influenzae* to human oropharyngeal cells may be inhibited by administering via a nasal passageway, or as a throat spray, a formulation containing a therapeutically effective amount of at least one of the forms of human β -casein identified in the preceding paragraph. Such a nasally administered formulation may be in the form of either drops or a spray.

The enteral, throat spray and nasal products and methods are believed to be effective in inhibiting the attachment of *H. influenzae* to human cells because the interaction of the human β -casein and *H. influenzae* is believed to occur in the nasopharynx via direct contact rather than following digestion and absorption of the β -casein.

It is believed that the above identified forms of human β -casein may be incorporated into any standard or specialized enteral liquid nutritional product containing at least one protein not found in human milk, such as bovine milk based or soy based infant formulas, and other beverages consumed by young children. In a preferred embodiment no proteins or hydrolysates thereof found in human milk, other than β -casein, are contained in the liquid enteral nutritional product. Such a product has utility in the treatment and prevention of otitis media in human infants.

While preferred embodiments of the invention have been disclosed, it

will be apparent to those skilled in the art that various changes and modifications may be made therein without deviating from the spirit or scope of this invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Abbott Laboratories
- (ii) TITLE OF INVENTION: Inhibition of Attachment of H. Influenzae to Human Cells
- (iii) NUMBER OF SEQUENCES: 5
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb storage
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: MS-DOS Version 6.21
 - (D) SOFTWARE: WordPerfect Version 6.0a
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 - (A) APPLICATION NUMBER: US 08/249,556
 - (B) FILING DATE: 26-MAY-1994
 - (A) APPLICATION NUMBER: US 08/249,584
 - (B) FILING DATE: 26-MAY-1994
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (614) 624-3774
 - (B) TELEFAX: (614) 624-3074
 - (C) TELEX: None
- (2) INFORMATION FOR SEQ ID NO: 1
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1065 base pairs
 - (B) TYPE: Nucleic acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Unknown
 - (ii) MOLECULE TYPE: Cloned cDNA representing the product of a human genomic DNA segment
 - (A) DESCRIPTION: Human milk β eta-casein
 - (iii) HYPOTHETICAL:
 - (iv) ANTI-SENSE:
 - (v) FRAGMENT TYPE:
 - (vi) ORIGINAL SOURCE: Human
 - (A) ORGANISM: Homo sapiens
 - (B) STRAIN:

- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE: Adult
- (E) HAPLOTYPE:
- (F) TISSUE TYPE: Mammary gland
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE: Human Mammary Gland
 - (A) LIBRARY:
 - (B) CLONE:
- (viii) POSITION IN GENOME:
 - (A) CHROMOSOME/SEGMENT:
 - (B) MAP POSITION:
 - (C) UNITS:
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis
 - (D) OTHER INFORMATION: The encoded product of nucleotide SEQ ID NO: 1 is the human milk protein, β -casein.
- (x) PUBPUBLICATION INFORMATION:
 - (A) AUTHORS: B. Lonnerdal, et al.
 - (B) TITLE: Cloning and sequencing of a cDNA encoding human milk beta-casein.
 - (C) JOURNAL: Federation European Biochemical Society Letters

- (D) VOLUME: 269
- (E) ISSUE:
- (F) PAGES: 153 - 156
- (G) DATE: 1990
- (H) DOCUMENT NUMBER:
- (I) FILING DATE:
- (J) PUBLICATION DATE:
- (K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

CGG	ATG	AAG	GTC	CTC	ATC	CTC	GCC	TGC	CTG	GTG	GCT	CTT	GCT	CTT	45
GCA	AGG	GAG	ACC	ATA	GAA	AGC	CTT	TCA	AGC	AGT	GAG	GAA	TCT	ATT	90
ACA	GAA	TAC	AAG	AAA	GTT	GAG	AAG	GTT	AAA	CAT	GAG	GAC	CAG	CAG	135
CAA	GGA	GAG	GAT	GAA	CAC	CAG	GAT	AAA	ATC	TAC	CCC	TCT	TTC	CAG	180
CCA	CAG	CCT	CTG	ATC	TAT	CCA	TTC	GTT	GAA	CCT	ATC	CCC	TAT	GGT	225
TTT	CTT	CCA	CAA	AAC	ATT	CTG	CCT	CTT	GCT	CAG	CCT	GCT	GTG	GTG	270
CTG	CCT	GTC	CCT	CAG	CCT	GAA	ATA	ATG	GAA	GTC	CCT	AAA	GCT	AAA	315
GAC	ACT	GTC	TAC	ACT	AAG	GGC	AGA	GTG	ATG	CCT	GTC	CTT	AAA	TCT	360
CCA	ACG	ATA	CCC	TTT	TTT	GAC	CCT	CAA	ATC	CCA	AAA	CTC	ACT	GAT	405
CTT	GAA	AAT	CTG	CAT	CTT	CCT	CTG	CCT	CTG	CTC	CAG	CCC	TTG	ATG	450
CAG	CAG	GTC	CCT	CAG	CCT	ATT	CCT	CAG	ACT	CTT	GCA	CTT	CCC	CCT	495
CAG	CCC	CTG	TGG	TCT	GTT	CCT	CAG	CCC	AAA	GTC	CTG	CCT	ATC	CCC	540
CAG	CAA	GTG	GTG	CCC	TAC	CCT	CAG	AGA	GCT	GTG	CCT	GTT	CAA	GCC	585
CTT	CTG	CTC	AAC	CAA	GAA	CTT	CTA	CTT	AAC	CCC	ACC	CAC	CAG	ATC	630
TAC	CCT	GTG	ACT	CAG	CCA	CTT	GCC	CCA	GTT	CAT	AAC	CCC	ATT	AGT	675
GTC	TAA	GAA	GAT	TTC	AAA	GTT	AAT	TTT	CCC	TCC	TTA	TTT	TTG	AAT	720
TGA	CTG	AGA	CTG	GAA	ATA	TGA	TGC	CTT	TTC	CGT	CTT	TGT	ATC	ACG	765
TTA	CCC	CAA	ATT	AAG	TAT	GTT	TGA	ATG	AGT	TTA	TAT	GGA	AAA	AAT	810
GAA	CTT	TGT	CCC	TTT	ATT	TAT	TTT	ATA	TAT	TAT	GTC	ATT	CAT	TTA	855
ATT	TGA	AAT	TTG	ACT	CAT	GAA	CTA	TTT	ACA	TTT	TCC	AAA	TCT	TAA	900
TTC	AAC	TAG	TAC	CAC	AGA	AGT	TCA	ATA	CTC	ATT	TGG	AAA	TGC	TAC	945
AAA	CAT	ATC	AAA	CAT	ATG	TAT	ACA	AAT	TGT	TTC	TGG	AAT	TGT	GCT	990
TAT	TTT	TAT	TTC	TTT	AAG	AAT	CTA	TTT	CCT	TTC	CAG	TCA	TTT	CAA	1035
TAA	ATT	ATT	CTT	AAG	CAT	AAA	AAA	AAA	AAA						1065

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 105 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
 - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
 - (A) ORGANISM:
 - (B) STRAIN:
 - (C) INDIVIDUAL ISOLATE:
 - (D) DEVELOPMENTAL STAGE:
 - (E) HAPLOTYPE:
 - (F) TISSUE TYPE:
 - (G) CELL TYPE:
 - (H) CELL LINE:
 - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
 - (A) LIBRARY:
 - (B) CLONE:
- (viii) POSITION IN GENOME:
 - (A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

(D) OTHER INFORMATION: The synthetic oligonucleotide assures adaptation of translation sequence to E. coli codon usage.

(x) PUBPUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: Expression of Human Milk β -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.

(C) JOURNAL: Protein Expression and Purification

(D) VOLUME: 4

(E) ISSUE:

(F) PAGES: 373 - 381

(G) DATE: 1993

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

CTG CAG AAT TCA TAT GCG TGA AAC CAT CGA ATC CCT GAG CTC GAG	45
CGA AGA ATC GAT CAC CGA ATA CAA AAA AGT TGA AAA AGT TAA ACA	90
CGA GGA CCA GGA TCC	105

(2) INFORMATION FOR SEQ ID NO: 3

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 71 base pairs
 - (B) TYPE: Nucleic acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE: Synthetic oligonucleotide
 - (A) DESCRIPTION:
- (iii) HYPOTHETICAL:
- (iv) ANTI-SENSE:
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence
 - (A) ORGANISM:
 - (B) STRAIN:
 - (C) INDIVIDUAL ISOLATE:
 - (D) DEVELOPMENTAL STAGE:
 - (E) HAPLOTYPE:
 - (F) TISSUE TYPE:
 - (G) CELL TYPE:
 - (H) CELL LINE:
 - (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
 - (A) LIBRARY:
 - (B) CLONE:
- (viii) POSITION IN GENOME:

24

- (A) CHROMOSOME/SEGMENT:
- (B) MAP POSITION:
- (C) UNITS:
- (ix) FEATURE:
 - (A) NAME/KEY:
 - (B) LOCATION:
 - (C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis
 - (D) OTHER INFORMATION: The synthetic oligonucleotide encodes the carboxy-terminal end and translation stop.
- (x) PUBPUBLICATION INFORMATION:
 - (A) AUTHORS: L. Hansson, et al.
 - (B) TITLE: Expression of Human Milk β -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.
 - (C) JOURNAL: Protein Expression and Purification
 - (D) VOLUME: 4
 - (E) ISSUE:
 - (F) PAGES: 373 - 381
 - (G) DATE: 1993
 - (H) DOCUMENT NUMBER:
 - (I) FILING DATE:
 - (J) PUBLICATION DATE:
 - (K) RELEVANT RESIDUES:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

AGA TCT ACC CTG TGA CTC AGC CAC TTG CCC CAG TTC ATA ACC CCA
TTA GTG TCT AAT AAG GAT CCG AAT TC

45

71

(2) INFORMATION FOR SEQ ID NO: 4

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 68 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Synthetic oligonucleotide

(A) DESCRIPTION:

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence

- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY:
- (B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

(D) OTHER INFORMATION: Modified enterotoxin STII signal sequence.

(x) PUBUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: Expression of Human Milk β -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.

(C) JOURNAL: Protein Expression and Purification

(D) VOLUME: 4

(E) ISSUE:

(F) PAGES: 373 - 381

(G) DATE: 1993

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

CAT GAA AAA GAA TAT CGC ATT TCT TCT TGC ATC GAT GTT CGT TTT
TTC TAT TGC TAC AAA TGC ATA TG

45

68

(2) INFORMATION FOR SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Synthetic oligonucleotide

(A) DESCRIPTION:

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: Synthetic Oligonucleotide Sequence

- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY:
- (B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD: DNA sequencing and restriction analysis

(D) OTHER INFORMATION:

(x) PUBUBLICATION INFORMATION:

(A) AUTHORS: L. Hansson, et al.

(B) TITLE: Expression of Human Milk β -casein in Escherichia coli: Comparison of Recombinant Protein with Native Isoforms.

(C) JOURNAL: Protein Expression and Purification

(D) VOLUME: 4

(E) ISSUE:

(F) PAGES: 373 - 381

(G) DATE: 1993

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

CAT ATG CAC GTG AAA CCA TCG AAT CCC TGA GCT CGA G

CLAIMS:

1. A liquid enteral nutritional product comprising at least one protein not contained in human milk in combination with at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the attachment of *H. influenzae* to human cells.

2. A liquid enteral nutritional product according to claim 1 wherein the product is an infant formula.

3. A liquid enteral nutritional product according to claim 1 where in the human cells are oropharyngeal cells.

4. A liquid enteral nutritional product according to claim 2 wherein the human cells are oropharyngeal cells.

5. A liquid enteral nutritional infant formula comprising at least one protein not contained in human milk in combination with at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the attachment to human cells, said infant formula containing no other proteins which are found in human milk.

6. A liquid enteral nutritional formula according to claim 5 wherein the human cells are oropharyngeal cells.

7. A nasally administrable formulation comprising at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the attachment of *H. influenzae* to human oropharyngeal cells.

8. A throat spray formulation comprising at least one material selected from the group consisting of β -casein isolated from human milk, a

recombinant form of the β -casein contained in human milk and hydrolysates of both in a therapeutically effective amount which inhibits the attachment of *H. influenzae* to human oropharyngeal cells.

9. A method of inhibiting the attachment of *H. influenzae* to human cells by enterally ingesting a liquid nutritional product comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

10. A method of inhibiting the attachment of *H. influenzae* to human cells in a human infant by enterally feeding to said human infant an infant formula comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

11. A method of treating and preventing otitis media in a human by inhibiting the attachment of *H. influenzae* to human cells by feeding to said human an enteral nutritional product comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

12. A method of treating and preventing otitis media in a human infant by inhibiting the attachment of *H. influenzae* to human cells by feeding to said human infant an enteral formula comprising at least one protein not contained in human milk in combination with a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

13. A method of inhibiting the attachment of *H. influenzae* to human oropharyngeal cells by administering via a nasal passageway a formulation containing a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

14. A method of inhibiting the attachment of *H. influenzae* to human oropharyngeal cells by administering a throat spray formulation containing a therapeutically effective amount of at least one material selected from the group consisting of β -casein isolated from human milk, a recombinant form of the β -casein contained in human milk and hydrolysates of both.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/03789

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 A23L1/305 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 06308 (B.ANDERSSON ET AL.) 16 May 1991 cited in the application see the whole document ---	1-14
X	MICROB.PATHOG., vol.8, no.5, May 1990 pages 315 - 323 G.ANIASSON ET AL. 'Anti-adhesive activity of human casein against Streptococcus pneumoniae and Haemophilus influenzae' cited in the application see the whole document --- -/-	1-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

24 July 1995

Date of mailing of the international search report

01.08.95

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Theuns, H

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03789

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ADV.EXP.MED.BIOL., vol.310, 1991 pages 167 - 171 C.SVANBORG ET AL. 'ANTI-ADHESIVE MOLECULES IN HUMAN MILK' see the whole document ---	1-14
X	WO,A,91 08675 (C.W.SLATTERY ET AL.) 27 June 1991 see page 1 - page 3 ---	1-8
X	WO,A,94 06306 (NEW ZEALAND DAIRY RESEARCH INSTITUTE) 31 March 1994 see claims 38,39 ---	1-8
X	WO,A,93 04171 (SYMBICOM AKTIEBOLAG) 4 March 1993 see claims 40-44 ---	1-8
X	WO,A,93 04172 (SYMBICOM AKTIEBOLAG) 4 March 1993 see page 37 - page 42 -----	1-8

INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/US 95/03789

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106308	16-05-91	SE-B- 465109 AU-B- 633647 AU-A- 6647090 EP-A- 0454813 JP-T- 4503077 SE-A- 8903625	29-07-91 04-02-93 31-05-91 06-11-91 04-06-92 01-05-91
WO-A-9108675	27-06-91	AU-A- 7177391	18-07-91
WO-A-9406306	31-03-94	AU-B- 4987393 EP-A- 0661927	12-04-94 12-07-95
WO-A-9304171	04-03-93	WO-A- 9304172 EP-A- 0599978	04-03-93 08-06-94
WO-A-9304172	04-03-93	WO-A- 9304171 EP-A- 0599978	04-03-93 08-06-94

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/ 03789

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9-14 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.